Rationale development of novel activity probes for the analysis of human Cytochrome P450's

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Abstract: Identification and quantification of functional cytochromes P450 (CYP450s) in biological samples is proving important for robust analyses of drug efficacy and metabolic disposition. In this paper, a novel CYP450 activity-based probe has been rationally designed and synthesised, demonstrating selective binding of CYP450 isoforms. The dependency of probe binding upon the presence of NADPH is permissive of selective detection of functionally active CYP450, allowing detection and analysis of these enzymes using biochemical and proteomic methodologies and approaches.

Cytochrome P450s (CYP450s) constitute a large family of haem-centred enzymes, with fundamental roles in the biotransformation of endogenous (steroid hormones, fatty acids, prostaglandins) and exogenous molecules (drugs, environmental chemicals, agrochemicals). As a direct result of their importance, particularly in xenobiotic and drug metabolism,[1] a great deal of research has been conducted into the roles, identification of their sequences and their catalytic mechanism.[2-4] Whilst a number of CYP450s, particularly human liver CYP450s and extra-hepatic CYP450s (i.e. CYP1A1, CYP1B1 and CYP2W1) have been the subject of intense investigation,[5-7] much is still to be learnt from these mixed-function oxidases. This reflects the difficulties associated with studying these enzymes as (i) they are encoded by large gene families and their functions cannot be predicted from their gene sequence, (ii) they are difficult to assay, isolate and purify, so classical biochemical methods are often ineffectual in identifying enzymes of interest, (iii) these proteins are membrane bound and often dependent on co-enzymes and co-factors, making them difficult to express as a functional enzymes in cellular systems, and (iv) polymorphisms and epigenetic regulation alter their expression and functional activity. As a result, new approaches to identifying, evaluating and quantifying functionally active CYP450s are of the upmost importance. One such approach is activity-based protein profiling, which involves 'tagging' the functional protein with a selective small molecule affinity probe, through covalent attachment (Figure 1, pathway B)[8-13]

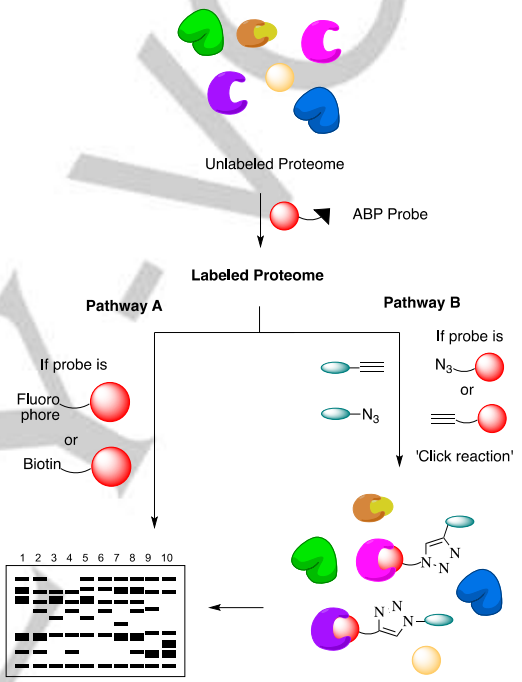


Figure 1: SDS-PAGE activity based protein profiling, directly with appropriately functionalised probes (pathway A) or orthogonally utilising 'click chemistry' (pathway B).

Whilst CYP450 probes developed to date exhibit the ability to bind these enzymes and thus indicate potential utility, several failed to demonstrate reactivity as a consequence of steric demands of the probe, and the majority lack differential or selective affinity for functional CYP450.[14-18] As a result, there is a requirement for new activity-based probes to study and identify this enzyme class. Here in, we report the synthesis of a small group of rationally designed activity-based probes, identifying benzofurans as suitable pharmacophores, for this purpose. In addition, evaluation of the probes efficiency and ability to selectively label CYP450s was achieved using well-established biochemical techniques, such as enzyme kinetics, immunoblotting, and proteomic mass spectrometry.

Results and discussion

The probe designs were inspired from the natural product furanocoumarin family, which are known irreversible inhibitors of cytochrome P450s.[14-17] Our rationale focused on the structural elements required for a probe to function

efficiently and most importantly, selectively against cytochrome P450s. Furanocoumarins inhibit cytochrome P450s following oxidation of the furan moiety to yield a reactive furan epoxide, which undergoes nucleophilic attack from a protein amino acid side chain to covalently bind the apoprotein. Therefore a rational disconnection approach identified two structural analogues **7** and **8**, with both retaining the furan moiety and a position for functionalization with a reporter group, whilst **8** possesses an additional aromatic ring to aid for greater selectivity in the active site (Figure 2). A third probe **9** was designed from coumarin, a known substrate for cytochrome P450s,[19, 20] to understand the requirement of the lactone fragment present in furanocoumarins. Coumarin is hydroxylated in the 7-position so it was envisioned, by installing a chloromethyl group in the 6-position, the hydroxylated product would undergo rearrangement in the active site producing an *ortho*-quinone methide **5** which would undergo attack from a protein amino acid side chain, covalently linking the probe and enzyme (Figure 2).

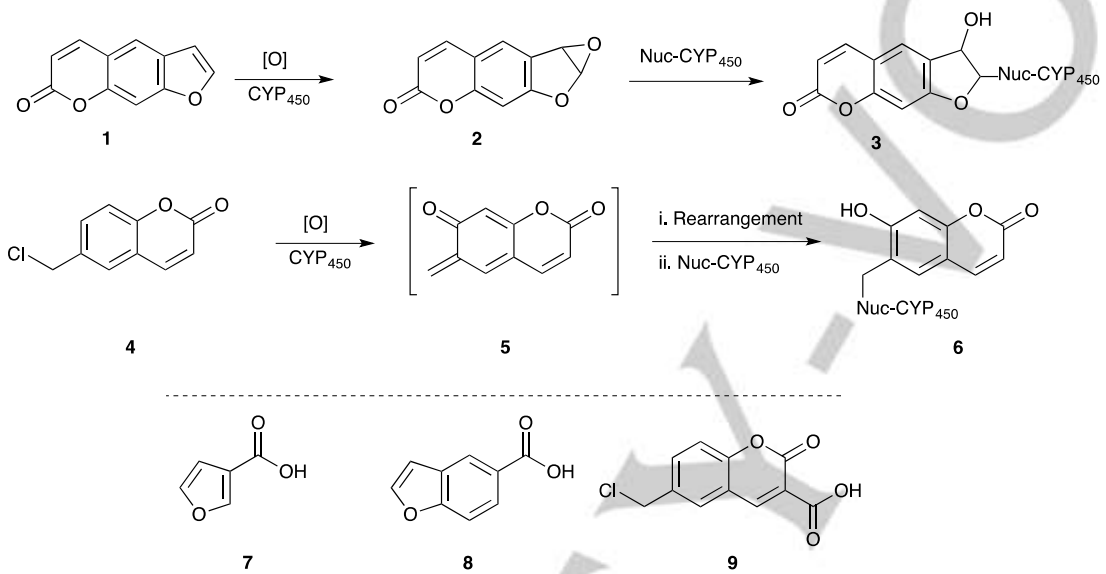
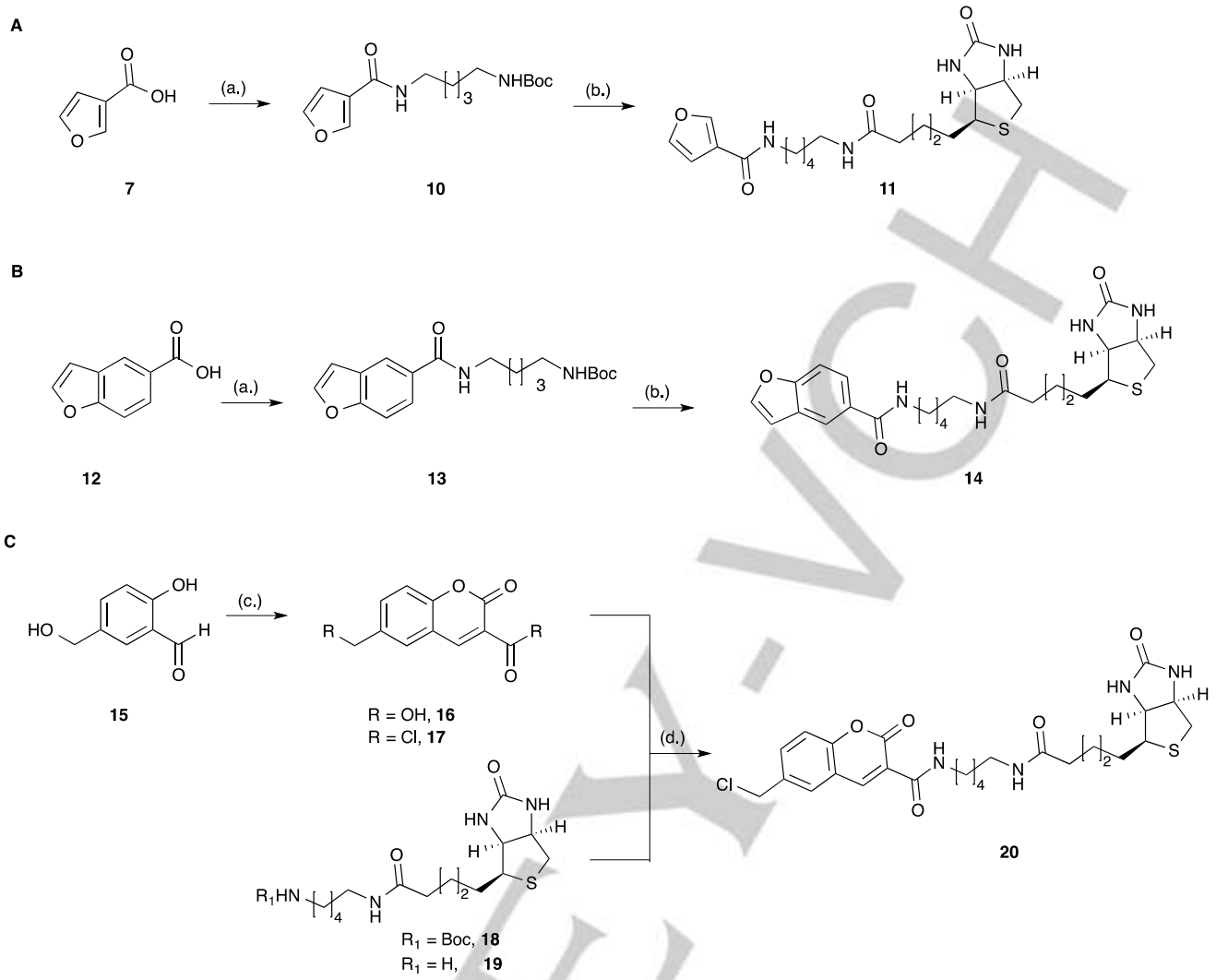


Figure 2: Mechanism of furanocoumarin inhibition; structural analogue for probe synthesis

Synthesis of the furan and benzofuran probes **11** and **14** was achieved in a linear sequence beginning with the addition of mono-protected cadaverine[21, 22] to the activated carboxylic acids **7** and **12**. Use of the reactive acid chloride of **7** led to satisfactory but low yielding amide **10**, presumably due to the instability of the acid chloride. Notwithstanding this, expedient synthesis of probes **11** and **14** is achieved through TFA deprotection of the amides **10** and **13** followed by acylation with biotin-NHS. Synthesis of probe **20** was undertaken with a divergent approach starting from 2-hydroxybenzaldehyde. Following a literature procedure,[23] a hydroxymethyl group was introduced with formaldehyde and conc. HCl to produce diol **15**, with subsequent cyclisation of the aldehyde and phenolic hydroxyl group with Meldrums acid provided the coumarin architecture **16**. Following a literature procedure[24] biotin was activated as the mixed anhydride, instead of the HOBt ester, and extended with mono-protected cadaverine[21, 22] providing **18** in good yield. Finally, **16** and **18** were joined through activation of the carboxylic acid moiety, and concomitant conversion of the hydroxylmethyl group to chloride, with thionyl chloride and deprotection of **18** with TFA to produce **20** in good yield over 3 steps (Scheme 1).



Scheme 1: *Reagents:* (a.) (i.) $SOCl_2$, DMF (cat.); (ii.) N-Boc-1,5-diaminopentane, DIPEA in DCM; (b.) (i.) **10** or **13**, TFA, DCM; (ii.) Biotin-NHS, Et_3N, DMF (c.) Meldrums acid, Pyridine, DCM, Δ; (d.) (i.) **16**, $SOCl_2$, Δ; (ii.) **18**, TFA, DCM; (iii.) **17** + **19**, *xs.* Et_3N

Biological investigations

The ability and potential of probes **11**, **14** and **20** to bind and inactivate CYP450 was evaluated using the Vivid assay,[25] for two recombinant enzymes; CYP3A4 a central component of many biotransformation processes known to be inhibited by furanocoumarins, and CYP1A2 that has an unknown endogenous substrate but broad exogenous substrate scope. [14, 26] Initially, varying concentrations of methanol (**11** and **14**) and DMSO (**20**) solvent controls were assessed in the assay to determine if they had any deleterious effects. Unsurprisingly, DMSO was observed to be very toxic to the CYP3A4 assay, resulting in 50 % inhibition at 0.5 % DMSO which prevented the measurement of **20**, which was only soluble in DMSO.[27, 28] The CYP3A4 assay was also sensitive to methanol, but to a lesser extent and could be used up to concentrations of 10% in combination with water to dilute **11** and **14**.[29] The CYP1A2 assay, by contrast, was tolerant of DMSO to 2% as well as methanol to 10%. Consequently, dilutions of **11** and **14** in 4-10% methanol/water for both CYP450s and dilutions of **20** in 4% methanol/2% DMSO for CYP1A2 enabled determination of IC_{50} values for these enzymes.[29] Probes **11**, **14** and **20** exhibited no significant inhibition of CYP1A2 activity at concentrations up to 2 mM, whereas **11** and **14** obtained inhibition of 230 and 90 μM respectively against CYP3A4 (**Error! Reference source not found.**). In addition, the K_{inact} and the K_i were determined for **14**, to be 0.042 (min^{-1}) and 0.5 mM respectively (Figure 3).

Probe	CYP3A4 inhibition (IC_{50}; μM ±S.D.)	CYP1A2 inhibition (IC_{50}; μM ±S.D.)
11	230 ± 141	>2000
14	90±7	>2000
20	ND	>2000

Table 1: IC_{50} values for cytochrome P450 3A4 (n = 2 measurements) and 1A2 (n = 3 measurements), ND – not determined

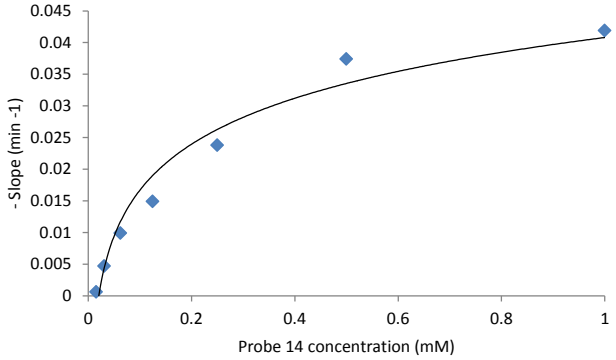


Figure 3. K_{inact} measurement of **14** for CYP3A4

Having established that **11** and **14** are capable of inhibiting CYP3A4, their ability to act as a probe and identify the enzyme-probe complex was assessed. As **11** and **14** possess a biotin moiety, well-established streptavidin identification was utilised. Firstly, incubation of **11** and **14** with bactosomes, followed by streptavidin blotting revealed that both **11** and **14** are activated by CYP3A4 and appear to covalently label the proteome, however multiple bands were detected with both probes.[29] These extra bands maybe the result of several factors, such as CYP3A4 aggregates and degradation products resulting from over expression of the gene in E.coli, or binding to E.coli proteins. The greater degree to which **11** labels the proteome suggests it maybe released from the active site as the long lived ene-dial intermediate, formed from rearrangement of the furan epoxide (*cf.* **2**), whilst **14** is a benzofuran which possesses an aromatic ring, increasing its binding affinity to the active site whilst also producing a more reactive benzofuran dioxetane, making labelling of the CYP450 more competitive. [30-33][34]

As a result, subsequent studies were conducted with **14** as fewer off-target binding events were observed. Firstly, incubation of **14** with baculosomes, followed by desalting of the sample to remove free probe before binding to streptavidin magnetic beads and SDS PAGE analysis of the bound proteins was performed (Figure 4A). Pleasingly, **14** revealed a distinguishable strong band at ~55 kDa on staining with Coomassie-Blue, however additional bands were still detected. Subsequently, **14** was treated under the same conditions as before except were visualised by Western blotting with horseradish peroxidase-conjugated Streptavidin and ECLplus detection (Figure 4B). Satisfyingly, **14** produced a band at 55 kDa, indicative of binding and enrichment of CYP3A4.

Having demonstrated that **14** was capable of identifying a band at approximately 55 kDa and with low off-target binding, attention turned to identifying the attached protein. Excision, digestion and analysis of the 55 kDa band from the Coomassie Blue stained gel

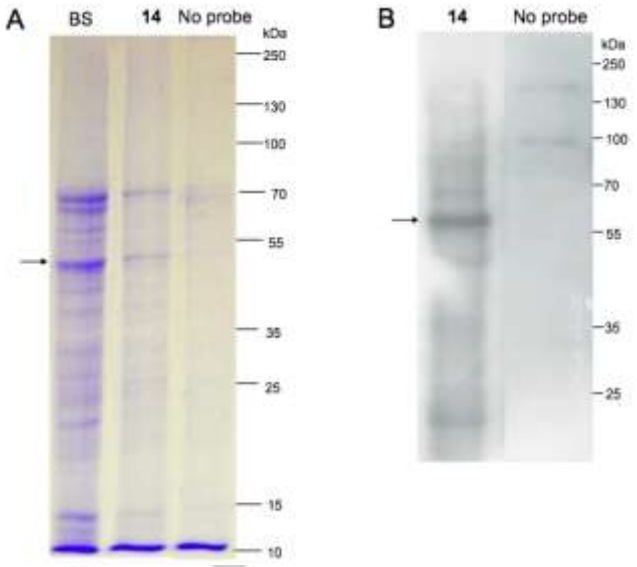


Figure 4: A. Coomassie Blue-stained SDS polyacrylamide gel of CYP3A4 complexed with **14** and recovered from streptavidin magnetic beads. BS = baculosome CYP3A4 starting material; B. Streptavidin blot of **14**, with no probe control incubated with CYP3A4 baculosomes**.**

by mass spectrometry identified the protein to be CYP3A4 by peptide mass fingerprinting (Mascot score = 110, with 26 matched peptides, MS threshold >56 was indicative of extensive homology) and MS/MS analysis of MH^+ = 1812.039 (score = 89, MS/MS threshold >30 was indicative of extensive homology), 1457.944 (score = 47) and 1960.278 (score = 69).

Satisfied with these results, attention turned to the selectivity of **14** against an array of bactosome-expressing CYP450s using streptavidin blotting. Probe **14** formed clear NADPH–dependent complexes with CYP1B1, CYP2B6 and CYP3A4, and to a lesser extent CYP2C9. The promiscuity of CYP450s is not unexpected, as they have been implicated in the metabolism of a variety of xenobiotic compounds in human liver.[35-37] The level of selectivity displayed by **14** in binding to CYP450s in an NADPH-dependant manner with little to no binding observed in its absence is exemplary (Figure 5A). Using CYP3A4 as an example, the time- and NADPH-dependent binding of **14** was shown to occur within 1 minute (Figure 5B).

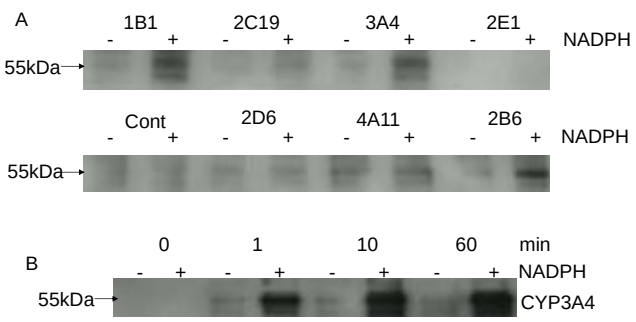


Figure 5. A. Streptavidin blot of probe **14** incubated with bactosomes expressing a specific CYP450 isoform in the presence or absence of NADPH. B. Time course of **14** reaction with CYP3A4

In conclusion, a small group of rationally designed probes have been synthesised, with a benzofuran probe 14 demonstrating respectable inhibitory activity against CYP3A4 (IC_{50} = 90 μM). In addition, **14** was able to identify CYP3A4 utilising well-established biochemical techniques, immunoblotting and mass spectrometry. There have been a number of studies to explore the binding of xenobiotics or activity based probes to cytochrome P450s, founded on coumarin and furan scaffolds. Furan is an established toxicant in liver and a screen of recombinant CYPs identified that CYP2E1 and CYP2D6 are enzymes responsible for its metabolism, with limited activity from CYP3A4.[38] There was inhibition of CYP3A4 activity in the Vivid assay by probe **11** (furan derivative) which may be due to bioactivation of the furan moiety resulting in the production of *cis*-2-butene-1,4-dial which in turn reacts with lysine side chains and N-termini of proteins.[30] Coumarin and benzofuran derivatives (related to naturally occurring compounds in grapefruit juice) were synthesised and analysed for CYP3A4 inhibition with the latter found to be the most potent.[39] Our benzofuran **14**, exhibited the most effective NADPH-dependent binding to CYP3A4 compared to other CYPs tested and consequently shows great promise for further investigation. Given the limited functionalization of **14**, the level of selectivity demonstrated in binding CYP450s in an NADPH-manner was exemplary. In this respect, it is pertinent to note that a number of early CYP450 probes demonstrate similar selectivity for a range of CYP450s.[18] Work is currently in progress to further optimise the selectivity of **14** and extend the library of probes to new CYP450s.

Experimental Section

All air and/or moisture sensitive reactions were carried out under an argon atmosphere. Solvents were purified and dried following established protocols. Petrol refers to petroleum spirit boiling in the 40-60 °C range. Ether refers to diethyl ether. All commercially available reagents were used as received unless otherwise stated. Flash column chromatography was performed according to the method of Still *et al* using 200-400 mesh silica gel.[40] Yields refer to isolated yields of products of greater than 95 % purity as determined by ^{1}H + ^{13}C NMR spectroscopy. Melting points were determined using Gallenkamp melting point apparatus and are uncorrected. Infrared spectra were recorded using a Diamond ATR (attenuated total reflection) accessory (Golden Gate) on a Perkin-Elmer FT-IR 1600 spectrometer. Unless otherwise stated ^{1}H NMR spectra were recorded in $CDCl_3$ on Varian Mercury-200, Varian VXR-400, Bruker Avance-400, Varian Inova-500, Varian VNMRS-700, and are reported as follows; chemical shift δ (ppm) (number of protons, multiplicity, coupling constant *J* (Hz), assignment). Residual protic solvent $CHCl_3$ (δ_H = 7.26) was used as the internal reference. ^{13}C NMR spectra were recorded at 63 MHz or 126MHz, using the central resonance of $CDCl_3$ (δ_C = 77.0 ppm) as the internal reference. All ^{13}C spectra were proton decoupled. Assignment of spectra was carried out using DEPT, COSY, HSQC, HMBC and NOESY experiments. High resolution accurate mass measurement was performed on a LTQ FT mass spectrometer (ThermoFinnigan Corporation) using flow-injection electrospray ionisation.

Standard procedure for the formation of 10 and 13

The carboxylic acid **7** or **12** (1 equiv.) was treated with an excess of thionyl chloride and a drop of DMF. After stirring for the reported time and temperature, the solvent was removed *in vacuo* and the resultant material was dissolved and evaporated with DCM (3 x 10 ml) to ensure excess thionyl chloride removal. The resultant material was dissolved in DCM, treated with *N*-Boc-cadaverine (1.2 equiv.) and DIPEA (4 equiv.) and stirred for the reported time and temperature. The reaction was then poured into sat. aq. NH_4Cl (10 ml), dried with $MgSO_4$, filtered and evaporated *in vacuo*.

***N*-Boc-(5-aminopentyl)-3-furancarboxamide 10**

Following the standard procedure, 3-furoic acid **7** (100 mg, 0.9 mmol) was transformed to a brown solid **10** (153 mg, 58 %); R_f 0.6 (*n*-hexane/EtOAc 1:1); δ_H (400 MHz, $CDCl_3$) 7.96 (1H, dd, *J* 1, Ar-*H*), 7.44 (1H, dd, *J* 2, Ar-*H*), 6.67 - 6.62 (1H, m, Ar-*H*), 5.99 (1H, bs, N*H*), 4.62 (1H, bs, N*H*Boc), 3.44 - 3.39 (2H, q, *J* 8, CH_2), 3.15 (2H, m, CH_2), 1.67 – 1.46 (6H, m, CH_2), 1.45 (9H, s, NHBoc); δ_C (101 MHz, $CDCl_3$) 162.7 (*C*=O), 156.2 (*C*=O), 144.7 (Ar-*C*), 143.7 (Ar-*C*), 122.6 (*ipso*-Ar-*C*), 108.3 (Ar-*C*), 39.3 (*C*H_2), 29.8 (*C*H_2), 29.2 (*C*H_2), 28.4 (Boc), 23.9 (*C*H_2); *m/z* (ES^+) 615.2 ($2M+Na^+$), 319.6 (MNa^+), 297.2 (MH^+)

***tert*-Butyl 5-(benzofuran-5-carboxamido)pentylcarbamate 13**

Following the standard procedure, benzofuran-5-carboxylic acid **12** (100 mg, 0.62 mmol) was transformed to a cream solid **13** (73 mg, 34 %); R_f 0.8 (*n*-hexane/EtOAc 1:1); υ_{max} (ATR) 3372 (NH), 3328 (NH), 2932, 2870, 1685 (C=O), 1628 (C=O), 1522, 1473, 1365, 1164, 1136, 1115, 1044, 1023, 1012, 949, 908, 883, 847, 821 cm^{-1}; δ_H (700 MHz, $CDCl_3$) 8.06 (1H, d, *J* 2, Ar-*H*), 7.72 (1H, dd, *J* 9, 2, Ar-*H*), 7.68 (1H, d, *J* 2, Ar-*H*), 7.52 (1H, d, *J* 9, Ar-*H*), 6.82 (1H, dd, *J* 2, 1, Ar-*H*), 6.24 (1H, bs, N*H*), 4.57 (1H, bs, N*H*), 3.49 (2H, q, *J* 6, CH_2), 3.15 – 3.12 (2H, m, CH_2), 1.69 – 1.64 (2H, m, CH_2), 1.58 – 1.51 (4H, m, CH_2), 1.45 – 1.41 (9H, s, NHBoc); δ_C (700 MHz, $CDCl_3$) 167.0 (*C*=O), 156.5 (*ipso*-Ar-*C*), 156.1 (*ipso*-Ar-*C*), 146.2 (Ar-*C*), 130.0 (*ipso*-Ar-*C*), 127.5 (*ipso*-Ar-*C*), 123.3 (Ar-*C*), 120.6 (Ar-*C*), 111.4 (Ar-*C*), 107.0 (Ar-*C*), 40.2 (*C*H_2), 40.0 (*C*H_2), 29.8 (*C*H_2), 29.3(*C*H_2), 28.4 (Boc), 24.0 (*C*H_2); *m/z* (ES^+) 369 (MNa^+), 244 (M-Boc^+); HRMS (ES^+) Found MNa^+, 369.1803 ($C_{19}H_{26}N_2O_4Na$ requires 369.1790).

Standard procedure formation of 11 and 14

The *N*-Boc protected furancarboxamide **10** or **13** (0.2 – 0.4 mmol) was dissolved in DCM (5 ml) and treated with an excess of TFA (*ca*. 1 ml). After stirring for the reported time and temperature, the solvent was removed *in vacuo* and the resultant material was dissolved and evaporated with DCM (3 x 10 ml) to ensure excess TFA removal. The resultant material was then dissolved in DMF, treated with Biotin-NHS (1.1 equiv.) and Et_3N (2.1 equiv.) and stirred for 4 hours. The solvent was then removed *in vacuo* and the resultant material flash chromatographed (DCM/methanol). Subsequent trituration with DCM (5 ml) produced the desired product.

_N_-[5-(furan-3-ylformamido)pentyl]-5-{2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl}pentanamide 11

Following the standard procedure, *N*-Boc protected furancarboxamide **10** (100 mg, 0.4 mmol) was transformed to a yellow solid **11** (113 mg, 79 %); R_f 0.3 (DCM/MeOH 9:1); δ_H (400 MHz, CD_3OD) 8.06 (1H, dd, *J* 1, Ar-*H*), 7.58 (1H, dd, *J* 2, Ar-*H*), 6.81 (1H, dd, *J* 2, 1, Ar-*H*), 4.53 (1H, ddd, *J* 8, 5, 1, CH*H*), 4.32 (1H, dd, *J* 8, 4, (C*H*H), 3.26 - 3.18 (4H, m, CH_2), 2.72 (2H, m, CH_2), 2.21 (2H, t, *J* 7, CH_2), 1.82 – 1.39 (12H, m, CH_2); *m/z* (ES^+) 445 (MNa^+), 424 (MH^+)

_N_-(5-(5-((3a*S*,4*S*,6a*R*)-2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentyl)benzofuran-5-carboxamide 14

Following the standard procedure, *tert*-Butyl 5-(benzofuran-5-carboxamido)pentylcarbamate **13** (100 mg, 0.29 mmol) was transformed to a white gummy solid **14** (100 mg, 73 %); R_f 0.3 (DCM/MeOH 9:1); υ_{max} (ATR) 3584 – 3072, 2940, 2868, 1697 (C=O), 1676 (C=O), 1635 (C=O), 1524, 1461, 1316, 1262, 1179, 1104, 1025, 826 cm^{-1}; δ_H (700 MHz, CD_3OD) 8.11 (1H, dd, *J* 2, 1, Ar-*H*), 7.84 (1H, d, *J* 2, Ar-*H*), 7.78 (1H, dd, *J* 9, 2, Ar-*H*), 7.56 (1H, dt, *J* 9, 1, Ar-*H*), 6.93 (1H, dd, *J* 2, 1, Ar-*H*), 4.47 (1H, ddd, *J* 8, 5, 1 C*H*), 4.27 (1H, dd, *J* 8, 5, C*H*), 3.42 – 3.39 (3H, t, *J* 8, CH_2), 3.21 – 3.15 (4H, m, CH_2), 2.90 (1H, dd, *J* 13, 5, CH_2), 2.68 (1H, d, *J* 13, CH_2), 2.17 (2H, m, CH_2), 1.70 – 1.55 (4H, m, CH_2), 1.45 – 1.38 (4H, m, CH_2); δ_C (700 MHz, CD_3OD) 176.1 (*C*=O), 170.5 (*C*=O), 166.1 (*C*=O), 158.0 (*ipso*-Ar-*C*), 147.9 (Ar-*C*), 130.9 (Ar-*C*), 128.9 (*ipso*-Ar-*C*), 124.8 (Ar-*C*), 121.9 (*ipso*-Ar-*C*), 112.1 (Ar-*C*), 108.1 (Ar-*C*), 63.4 (*C*H), 61.6 (*C*H), 56.9 (*C*H_2), 41.05, 41.0, 40.9, 40.2, 36.8, 30.11, 30.07, 29.7, 29.4, 26.9, 25.3; *m/z* (ES^+) 967 (2M+Na^+), 495 (MNa^+), 473 (MH^+); HRMS (ES^+) Found MNa^+, 495.2025 ($C_{24}H_{32}N_4O_4SNa$ requires 495.2042).

2-Hydroxy-5-(hydroxymethyl)benzaldehyde 15

A mixture of salicylaldehyde (9 ml, 84.5 mmol), formaldehyde (17 ml, 37 % aq., 200 mmol) and 42 ml of concentrated HCl was heated to 80 °C for 20 min. After cooling, the supernatent was removed by settling of the phases and the residual pink crystalline mass was taken up in 200 ml of H_2O. The suspension was refluxed for 30 min and then the supernatent was separated out after cooling. This step was repeated and the supernatants combined and placed at 4 °C to bring about crystallisation. Filtration of the solid material and washing with H_2O afforded the title compound as a white solid (1.7 g, 13 %); m.p. 105 - 107 °C; υ_{max} (ATR) 3418 – 3120 (broad – OH), 2876, 1651, 1578, 1481, 1454, 1376, 1358, 1307, 1274, 1187, 1145, 1017, 902, 839 cm^{-1}; δ_H (500 MHz, $CDCl_3$) 11.0 (1H s Ar-C*H*O), 9.92 (1H, s, Ar-O*H*), 7.59 (1H, d, *J* 2, Ar-*H*), 7.54 (1H, dd, *J* 9, 2, Ar-*H*), 7.0 (1H, d, *J* 9, Ar-*H*), 4.70 (2H, s, Ar-CH_2); δ_C (500 MHz, $CDCl_3$) 196.5 (*C*=O), 161.1 (*ipso*-Ar-*C*), 136.0 (Ar-*C*), 132.9 (Ar-*C*), 132.1 (Ar-*C*), 120.3 (*ipso*-Ar-*C*), 117.8 (Ar-*C*), 64.2 (*C*H_2); *m/z* (ES^-) 151 (MH^-); HRMS (ES^-) Found MH^-, 151.0391 ($C_8H_7O_3$ requires 151.0395).

6-(Hydroxymethyl)-2-oxo-2*H*-chromene-3-carboxylic acid 16

2-Hydroxy-5-(hydroxymethyl)benzaldehyde (1.2 g, 7.9 mmol) was dissolved in DCM and treated with Meldrums acid (1.4 g, 9.5 mmol) and pyridine (1.3 ml, 15.8 mmol). The solution was refluxed for 2 h, then after cooling the precipitate was collected by filtration and washed with DCM to afford the title compound as a pale yellow solid (1.2 g, 70 %); δ_H (400 MHz, DMSO) 8.43 (1H, s, Ar-*H*), 7.73 (1H, s, Ar-*H*), 7.58 (1H, dd, *J* 9, 2, Ar-*H*), 7.34 (1H, s, Ar-*H*), 4.55 (2H, s, Ar-CH_2); *m/z* (ES^+) 243 (MNa^+), 221 (MH^+); all data agree with those reported in the literature.[23]

6-(Chloromethyl)-2-oxo-*N*-(5-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentyl)-2*H*-chromene-3-carboxamide 20

6-(Hydroxymethyl)-2-oxo-2*H*-chromene-3-carboxylic acid **16** (50 mg, 023 mmol) was dissolved in thionyl chloride (4 ml) and refluxed for 2 h. At the same time, *tert*-butyl 5-(5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamido)pentylcarbamate[24] **18** (97 mg, 0.23 mmol) was dissolved in DCM (2 ml) and treated with TFA (2 ml). Evaporation of the two reactions to remove the thionyl chloride and TFA was followed by dissolution of the two reactions in DCM (2 ml). The crude 6-(chloromethyl)-2-oxo-2*H*-chromene-3-carbonyl chloride **17** was then added via cannulla to a mixture of crude *N*-(5-aminopentyl)-5-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide **19** and Et_3N (95 µl, 0.7 mmol) at room temperature. The reaction was stirred for 2 h and then evaporated. Flash chromatography (DCM, DCM/MeOH [99:1], [98:2], [95:5], [9:1]) followed by trituration with MeOH afforded the title compound as a white gummy solid (55 mg, 44 %); R_f 0.3 (DCM/MeOH 9:1); υ_{max} (ATR) 3512 – 3150, 2928, 2854, 1697 (C=O), 1653 (C=O), 1614 (C=O), 1573, 1536, 1419, 1246, 1168, 1021, 827 cm^{-1}; δ_H (500 MHz, DMSO) 8.80 (1H, s, Ar-*H*), 8.67 (1H, t, *J* 6, N*H*), 8.02 (1H, d, *J* 2, Ar-*H*), 7.80 (1H, dd, *J* 9, 2, Ar-*H*), 7.52 (1H, d, *J* 9, Ar-*H*), 6.42 (1H, s, N*H*), 6.35 (1H, s, N*H*), 4.84 (2H, s, Ar-CH_2), 4.29 (1H, m, C*H*), 4.15 (1H, m, C*H*), 3.09 – 3.00 (4H, m, CH_2), 2.80 (2H, dd, *J* 12, 5, CH_2), 2.64 (2H, m, CH_2), 2.02 (2H, t, *J* 6, CH_2), 1.62 – 1.23 (6H, m, CH_2), 1.15 (2H, t, *J* 6, CH_2); δ_C (500 MHz, DMSO) 172.6 (*C*=O), 163.4 (*ipso*-Ar-*C*), 161.6 (*C*=O), 161.0 (*C*=O), 154.2 (*C*=O), 147.5 (Ar-*C*), 135.39 (*ipso*-Ar-*C*), 135.20 (Ar-*C*), 130.9 (Ar-*C*), 120.4 (*ipso*-Ar-*C*), 119.2 (*ipso*-Ar-*C*), 117.4 (Ar-*C*), 61.7, 59.9, 56.1, 46.4, 45.7, 38.9, 35.9, 29.5, 29.4, 28.9, 28.7, 26.0, 24.5, 9.3; *m/z* (ES^+) 549 ([^{35}Cl]MH^+); HRMS (ES^+) Found [^{35}Cl]MH^+, 549.1928 ($C_{26}H_{34}{}^{35}ClN_4O_5S$ requires 549.1938).

Baculosome CYPs.

Baculosome CYP1A2 and CYP3A4 were purchased from ThermoFisherScientific, were used for the Vivid assays and streptavidin affinity enrichment.

Cytochrome P450 Fluorescent Assays

The Vivid® CYP3A4 assay (Life Technologies, Carlsbad, CA) was run according to the manufacturer's instructions. A master pre-mix was prepared comprising, 4500 µl of 100 mM potassium phosphate buffer, pH 8.0, 100 µl of Regeneration System (333 mM glucose-6-phosphate and 30 U/ml glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer, pH 8.0) and 400 µl of CYP3A4 BACULOSOMES® (microsomes from baculovirus-infected cells co-expressing human CYP3A4, NADPH-cytochrome P450 reductase and human cytochrome b5). An aliquot of master pre-mix (50 µl) was pre-incubated with 40 µl of either 10% methanol (control) or serial dilutions of 1 mM solutions of probes **11** or **14** in 10% methanol, in a 96-well plate (Nunc™, ThermoFisher Scientific) for 10 minutes at room temperature. The reaction was initiated with the addition of 10 µl of the substrate solution (920 µl of 100 mM PBS, pH 8.0, 30 µl NADP+ and 50 µl 3A4 substrate, 7-benzyloxy-methyloxy-3-cyanocoumarin). Final concentrations were as follows: CYP3A4 – 40nM, BOMCC – 10µM, $NADP^+$ - 30µM, G6P – 3.33mM, and G6PD – 0.3U/ml. The relative fluorescence units (RFU) readings were then taken continuously every minute for 30 minutes using the Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (Thermo Fisher Scientific, UK) and Ascent software version 2.6 (Thermo Lab systems, UK), with 390 nm and 460 nm, excitation and emission wavelengths respectively.

The assay for CYP1A2 was analogous to that described for CYP3A4. A master pre-mix was prepared comprising, 2800 µL of 10 mM PBS (pH 7.4) 100 µL of regeneration system and 100 µL of CYP1A2 BACULOSOMES® (microsomes from baculovirus-infected cells co-expressing human CYP1A2, NADPH-cytochrome P450 reductase and human cytochrome b5). An aliquot of master pre-mix (30 µL) was pre-incubated with 40 µl of each dilution of a probe for 10 minutes at 37°C with in the Fluoroskan Ascent FL Microplate Fluorometer and Luminometer. The reaction was initiated with addition of 30 µL of the CYP1A2 substrate solution (2940 µL of 10mM, PBS pH 7.4, 30 µL $NADP^+$ and 30 µL 7-ethoxymethoxy-3-cyanocoumarin) which was also pre-incubated at 37°C. Final concentrations were as follows: CYP1A2 – 10nM, EOMCC – 2µM, $NADP^+$ - 10µM, G6P – 3.33mM, and G6PD – 0.3U/ml. The fluorescent readings were taken in the similar way as described above. The effect of each probe was calculated by comparing relative fluorescent unit (RFU) with the control assay containing 40 µL of PBS instead of the probe. To investigate IC_{50} values for both assays, non-linear regression analysis was done using GraphPad Prism 6 software.

Probe-bactosome expressed CYP reactions

Human CYP1B1, 2B6, 2C19, 2D6, 2E1, 3A4 and 4A11, along with control bactosomes expressed in *Escherichia coli*, were purchased from Cypex (Dundee, UK) and used to screen streptavidin blots for specificity, reaction time and concentration (Figure 5). Each was co-expressed with human cytochrome P450 reductase, and CYP3A4 and 4A11 also contained cytochrome b5. The expression levels (nmoles or µg of CYP per mg of total protein) for each CYP were different for each product. Therefore, each CYP (1 µg, equivalent to 16 to 18 pmoles of CYP, Figure 5) was diluted with PBS to a concentration of 0.05 µg/µl. Control bactosomes were diluted in PBS to give a 4.25 µg/µl protein solution, which was equivalent to the average protein concentration for the CYP bactosomes used. Each experiment was performed in duplicate to confirm reproducibility. For CYP profiling, each reaction, NADPH (1 mM, Sigma, Poole, UK) or PBS for NADPH-negative controls, was added to a 0.05µg/µl of PBS solution of one of the CYPs or control bactosomes.

CYP 1B1, 2B6, 2C9, 2D6, 2E1, 3A4 and 4A11 were obtained from CYPEX as membrane suspensions containing 1nmol P450/ml at 580, 88, 306, 282, 370, 327 and 147 pmol/mg protein.

Probe **14** (5mM in PBS, 20 % methanol) was added to each reaction mixture (equivalent to final concentrations of 0.5 mM), incubated at room temperature for 60 minutes before the reactions were stopped by transfer to reducing SDS loading buffer. For a time course experiment, NADPH (1 mM, Sigma, Poole, UK) or PBS for a NADPH-negative control was added to a 0.05µg/µl of PBS solution of CYP3A4. An aliquot was removed from each reaction (representing T=0) and transferred to 4 µl of reducing SDS loading buffer. Probe **14** (5mM in PBS, 20 % methanol) was added to each reaction and aliquots removed at 1, 10 and 60 minutes. Each aliquot was transferred to reducing SDS loading buffer to stop the reaction.

SDS PAGE/Streptavidin-HRP blotting

Samples (16 – 18 pmol CYP) were applied to 10 % SDS-PAGE gels at 80 V (Bio-Rad Laboratories, Hemel Hempstead, UK) for 15 minutes then at 150 V for 60 minutes. Following this, the gel was transferred to a nitrocellulose membrane (GE Healthcare, Amersham, UK) at 58 mA (Bio-Rad Laboratories, UK) for two hours. After transferring, membranes were blocked by 5 % milk powder at 4 ^oC overnight followed by a further 30 minutes at room temperature. After three washes with TBS-Tween-20 (0.05 %) for 15 minutes, the membranes were incubated with horseradish peroxidase-conjugated Streptavidin (Abcam, Cambridge, UK) at a dilution of 1:500 in 5 % blocking buffer for 1 hour at room temperature. Membranes were washed a further three times, each time incubated for 15 minutes at room temperature. ECL plus (GE Healthcare) was used for Streptavidin detection followed by exposure using imaging film (GE Healthcare).

Affinity purification using immobilised probes and mass spectrometric analysis

CYP3A4 baculosomes, equivalent to 5µg of recombinant CYP3A4, were incubated with probe **14** or PBS for 15 minutes at 37 ^oC. Each reaction mixture was desalted in NAP-

5 size exclusion columns (GE Healthcare, UK), equilibrated with 10mM ammonium bicarbonate, to remove free probe. The protein-probe complex, was lyophilised, resuspended in PBS incubated with bovine serum albumin pre-coated streptavidin Dynabeads (Life Technologies, Paisley). After three PBS, three 1M NaCl phosphate buffer and three 10mM ammonium bicarbonate, 10 % acetonitrile washes, the beads were resuspended in SDS PAGE Laemmli buffer and incubated at 90 ^{o}C for 15 minutes. The proteins released from the beads by the buffer were separated by SDS PAGE and stained with Coomassie Blue R-250 (Thermo Fisher Scientific). Bands of interest were excised from the gel and subject to in-gel digestion.[41] The resulting peptides were desalted in C_{18} ZipTips (Millipore) and analysed by MALDI MS in reflectron, positive ion mode (Ultraflex II, Bruker Daltonik, Bremen) for peptide mass fingerprinting and MS/MS for selected peptide fragmentation. The resulting spectra were searched using Mascot version 2.4 (Matrix Science, UK) against SwissProt version 2015_06 (Homo sapiens, 20207 sequences) to identify the proteins.

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Keywords: cytochrome P450 • activity based protein profiling • proteomics • benzofuran • activity based probes

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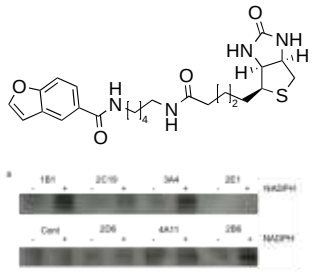

Identification of functional cytochrome P450 (CYP450) enzymes in primary tissues is important for the robust analysis of drug efficacy and metabolism. In this paper we describe the synthesis of a novel CYP450 activity-based probe, which demonstrates selective binding of human recombinant CYP450 isoforms.